PHYLOGENETIC ANALYSIS OF NORTH AMERICAN PLUMS (PRUNUS SECT. PRUNOCERASUS: ROSACEAE) BASED ON NUCLEAR LEAFY AND S6PDH SEQUENCES

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ABSTRACT

Phylogenetic analysis of DNA sequences from two low-copy nuclear genes (*LEAFY* and *s6pdh*) was conducted to investigate relationships among native plums of North America (*Prunus* sect. *Prunocerasus*). The cladograms resulting from maximum parsimony and Bayesian inference are poorly resolved and incongruent with clades based on chloroplast DNA sequences. Evidence from both nuclear genes confirms the placement of *P. texana* in sect. *Prunocerasus*. Sequences of *P. americana* and *P. mexicana* form a monophyletic group on the *s6pdh* cladogram, but most *P. americana* collections also have a second divergent sequence, which together form a clade sister to *P. subcordata*. The shared possession of a 250-bp insertion within *LEAFY* intron 2 supports a close relationship between *P. hortulana* and *P. murrayana*. *Prunus* alleghaniensis, *P. gracilis*, *P. maritima*, and *P. umbellata* form a paraphyletic group on the *LEAFY* cladogram, basal to a clade composed of *P. angustifolia* and *angustifolia*-like sequences of *P. rivularis* and *P. munsoniana*. Data from *LEAFY* and *s6pdh* suggest hybrid origins for *P. rivularis* and *P. munsoniana*, with *P. angustifolia* as one progenitor.

RESUMEN

Se realizó un análisis filogenético de secuencias de DNA de dos genes nucleares (LEAFY y s6pdh) para investigar las relaciones entre los ciruelos nativos de Norte América (Prunus sect. Prunocerasus). Los cladogramas que resultan de la máxima parsimonia e inferencia bayesiana tienen una resolución pobre y son incongruentes con clados basados en secuencias de DNA plastidial. Los resultados de ambos genes nucleares confirman la colocación de P. texana en la sect. Prunocerasus. Las secuencias de P. americana y P. mexicana forman un grupo monofilético en el cladograma de s6pdh, pero la mayor parte de las colecciones de P. americana tienen una segunda secuencia divergente, que juntas forman un clado hermano de P. subcordata. La posesión compartida de una inserción de 250-bp en el intrón 2 de LEAFY apoya una relación fuerte entre P. hortulana P. murrayana. Prunus alleghaniensis, P. gracilis, P. maritima, y P. umbellata forman un grupo parafilético en el cladograma de LEAFY, basal al clado compuesto por P. angustifolia y secuencias similares a angustifolia de P. rivularis y P. munsoniana. Los datoa de LEAFY y s6pdh sugieren origen híbrido para P. rivularis y P. munsoniana, con P. angustifolia como uno de los progenitores.

INTRODUCTION

Prunus is one of the larger genera of the Rosaceae with about 200 species, which are most abundant in the North Temperate zone but also range into the tropics and Southern Hemisphere (Rehder 1940; Robertson 1974). It is important commercially as the source of cherries, almonds, peaches, nectarines, apricots, and plums; numerous species are grown ornamentally for their beautiful early-spring flowers. The classification of Prunus has been debated from Linnaeus to the present day, with some botanists arguing for a broad concept of the genus that includes all of the fruits mentioned above (e.g., McVaugh 1951; Robertson 1974) and others dividing Prunus into a variety of segregate genera including Amygdalus, Armeniaca, Cerasus, Laurocerasus, Padus, and Persica (e.g., Iwatsuki et al. 2001; Flora of China Editorial Committee 2003). The most widely used classification has been that of Rehder (1940) who recognized Prunus in its broad sense with five subgenera.

At the species level there has been considerable taxonomic uncertainty among the North American plums (*Prunus* sect. *Prunocerasus*). In 1892 Bailey called *Prunocerasus* the "hardest puzzle in American pomology," a memorable phrase quoted by Shaw and Small (2004) more than a century later in reporting the use of multiple cpDNA sequences to bear on this enduring puzzle. Shaw and Small (2004) provided an

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extensive review and analysis of the taxonomic history of *Prunocerasus*, and they noted that the unresolved classification of North American plums is the result of interspecific similarities, intraspecific variation, and probably interspecific hybridization.

The earliest studies using DNA sequences to examine relationships in *Prunus* focused on a limited number of cultivated species (Badenes & Parfitt 1995; Uematsu et al. 1991). In 2001 two research groups sought to clarify phylogenetic relationships among the subgenera and numerous sections of *Prunus* by examining species on a worldwide scale (Bortiri et al. 2001; Lee & Wen 2001). Unfortunately relationships among the species of section *Prunocerasus* remained unresolved. Both groups used ITS sequences and found slight variation among the native plums included in their studies. Analyses by Bortiri et al. (2001) using chloroplast *trnL-trnF* sequences also revealed only slight variation among six North American species.

Rohrer et al. (2004) used microsatellite analysis in an attempt to find molecular markers more variable among species of *Prunocerasus* than ITS or *trn* sequences. However, all pairs of species in the study shared fewer than half of their alleles, indicating greater genetic divergence among the North American plums than expected given their similar ITS and *trn* sequences. Accessions of two or three individuals of the same species often did not cluster together, suggesting that microsatellites might be too variable to be informative in resolving species relationships in *Prunocerasus*. Seeking to test the hypothesis of monophyly for *Prunocerasus* and to resolve relationships among the species, Shaw and Small (2004, 2005) turned to seven noncoding regions in the chloroplast genome shown to be highly variable across a wide array of taxa (Shaw et al. 2005). Given their taxonomically broad sampling of species both within the section and across all of *Prunus*, Shaw and Small (2004, 2005) provided unequivocal support for the monophyly of sect. *Prunocerasus*. Within the section the analyzed sequences varied little among the species. They demonstrated strong support for *P. subcordata* as sister to the rest of *Prunocerasus*. An unexpected result was the inclusion of the hairy-fruited *P. texana* within *Prunocerasus* positioned between *P. subcordata* and the other species, which formed three primary clades with little resolution within each clade. Many of the species have exemplars on more than one clade, and the chloroplast haplotypes show greater correlation with geography than species limits.

Bortiri et al. (2002) demonstrated that the nuclear gene s6pdh was more variable among Prunus species than either ITS or trn and showed greater promise for resolving relationships in the genus. However, only three species of Prunocerasus were included in their analysis. In addition, Oh and Potter (2003) reported sequences from the second intron of the nuclear gene LEAFY were more phylogenetically informative than either ITS or cpDNA in the rosaceous genera Neillia and Stephanandra. In this study we explore the use of these low-copy nuclear genes for resolving relationships among the species of Prunocerasus. Specifically we expand the use of s6pdh to all commonly recognized species in the section and include sequences from the second intron of LEAFY.

MATERIALS AND METHODS

Plant Material.—We were able to obtain at least one accession from each of the 15 (or so) species of North American plums. The exact number of species is subject to the differences in opinion concerning species circumscriptions that animate taxonomic debate. Apricot (*P. armeniaca*), cherry plum (*P. cerasifera*), and two varieties of sand cherry (*P. pumila* var. pumila and *P. pumila* var. susquehanae) were used for outgroup comparison. In all, we analyzed 37 accessions of North American plums plus the four outgroup taxa (Table 1). Some specimens were collected from wild plant populations; others were collected from cultivated plants of the University of California, Davis campus, the USDA National Clonal Germplasm Repository in Davis, and the USDA Southeastern Fruit and Nut Research Station in Byron, GA. All sources of DNA are documented by voucher specimens deposited in the University of Wisconsin-Eau Claire herbarium (UWEC). We verified identifications against published descriptions and authentic herbarium material.

DNA Extraction, Amplification, and Sequencing.—Total DNA was isolated by grinding approximately 100 mg (wet weight) of fresh or frozen leaves in liquid nitrogen and using the DNeasy Plant Mini Kit (Qiagen) according to the standard protocol. We prepared PCR reactions in 50-µl volumes containing ~10 ng

Table 1. Plant material used in study (Haplotype letters correspond to those of Shaw and Small (2005) as determined from trnl-trnF spacer sequences. Vouchers are collectic deposited in UWEC. Key to source: state only = collected from wild; DPRU = USDA National Germplasm Repository in Davis, CA; SFNRS = USDA Southeastern Fruit an search Station in Byron, GA. Superscripted numerals preceding GenBank numbers individual clones, superscripted S and L indicate direct sequenced short (750bp) (1000 bp) fragments).

Haplotype	Species	Koucher	Source	GenBank Accession Numbers	
	Native American Taxa				updos
	P. alleghaniensis Marshall	10,467	Wichigan Teginan	EU050711	EU056679
	P. alleghaniensis Marshall	10,468		EU050712	EU056680
	ana Marshall (glabrou	10,495	Wisconsin	² EU047724, ⁴ EU047725	² EU056681, ³ EU056682
\	P. americana Marshall (glabrous)	O'	Wisconsin	1050713	23[
	P. americana Marshall (glabrous)	10,508	\circ		4EU0566
U	P. americana Marshall (hairy)	0,5	CA: DPRU 0544	² EU047726, ³ EU447296	
	americana Marshall (hairy)	10,607	Wisconsin	EU050715	² EU056688, ³ EU056689
()	americana N	10,684	Pennsylvania	EU050716	F
U	angr	10,519	Texas	EU050717	
U	P. angustifolia Marshall	0,5	CA: DPRU 1924	¹ EU047727, ² EU447297	EU056693
മ	genicul	10,609		EU050722	EU056698
U	Sept.	10,516	Exas	EU050723	EU056699
	P. hortulana L. H. Bailey	10,598	G 49	¹ EU047728, ⁴ EU047729	EU056700
	hori	9′0	GA: SFNRS	EU050724	EU056701
~	P. maritima Marshall	O'	CA: DPRU 1737	EU050725	EU056702
		0,5	Lexas Asas	EU050726	EU056703
	mexicana S.	0,51	Fxas	EU050718	EU056694
	P. mexicana S. Watson	0,5	Fexas	EU050719	EU056695
	mexicana S.	0,52	Texas	EU050727	EU056704
	mexicana	0,54	CA: UCD campus	EU050728	EU056705
	mexicana S. Watson	0,59	CA: DPRU 1368.1	EU050729	EU056706
	na W. Wight	10,513	Texas	- 5	¹ EU056707, ² EU056708, ³ EU056709
	munsoniana W. Wight & Hedric	0,59	CA: DPRU 0546	¹ EU047731, ⁴ EU047732	23EU056
					24EU056712
	munsoniana W. Wight	0	GA: SFNRS	¹ EU447298, ⁵ EU047733	EU056713
	munsoniana W. Wight & Hedric	0,67	Pennsylvania	¹ EU047744, ³ EU047745	
	murrayana E. J. P	0,52	Fxas		EU056715
	murayana	10,525	Texas	LEU050732, ⁵ EU050733	EU056716
	P. murrayana E. J. Palmer	10,526	EXAS	U0507	EU056717
	P. nigra Aiton	10,606	Wisconsin	EU050735	EU056718

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Haplotype	Species Native American Taxa		Source	GenBank Accession Numbers	we with the second seco
	P. nigra Aiton	10,633	Wisconsin	EU050736	EU056719
	P. rivularis Scheele		– exas	⁵ EU447299, ¹ EU047739, ² EU047740, ²¹ EU047734, ²⁴ EU047735	¹ EU056721, ²² EU056722
	P. rivularis Scheele		Lexas	² EU047741, ³ EU047736, ²² EU047742, ²⁴ EU447300, ²⁵ EU447301	² EU056723, ³ EU056724
	P. rivularis Scheele	10,528	Texas	4	² EU056725, ⁴ EU056726
U ∩	P. subcordata Benth.	10,595	CA: DPRU 2296	EU050739	EU056727
	P. texana D. Dietr.	10,685	- Lexas	F1050741	EU056729
U	P. umbellata Elliott	10,520	Texas:	EU050742	3EU056730, 4EU056731
	P. umbellata Elliott	10,620	GA: SFNRS	EU050743	EU056732
	Outgroups				
	P. armeniaca L.	10,589	CA: DPRU 1134	EU050720	EU056696
	P. cerasifera Ehrh.	10,541	California	EU050721	EU056697
	P. pumila L. var. pumila	10,611	Wisconsin	EU050737	EU056720
	P. pumila var. susquehanae (Willd.) H. Jaeger	10,445	Wisconsin	EU050740	EU056728

of genomic DNA, 1.25 units of *ExTaq* DNA polymerase (Takara), 1x PCR buffer, 200 μM dNTPs, 0.2 μM of each primer, and sterile water. Intron 2 of the nuclear gene *LEAFY* was amplified using primers modified from Oh and Potter (2003): LFY5: 5'-CAGAACATTGCCAAGGAGC-3', LFY4: 5'-GGCTTGTTGATGTAGCT-TGC-3'; and nuclear gene *s6pdh* from Exon 2 to Exon 6 was amplified using primers modified from Bortiri et al. (2002) plus a new primer (o): *s6pdh*-k: 5'-CAAGAGTGAAGCAGACGTTGG-3', *s6pdh*-h: 5'-AGACCA-ATGCTACGAACTAGGCCG-3', *s6pdh*-o: 5'-AGAATAAGGTGTTGGACATAGACG-3', *s6pdh*-p: 5'-AGAGTG-GTCCTGGATTTCTTATCTA-3'. Thirty-five cycles of three-step PCR, preceded by an initial melting step of 4 min at 94°C and concluded by a final extension of 7 min at 72°C, were carried out as follows: denaturation at 94°C for 30 sec, primer annealing at 52°C (*LEAFY*) or 54°C (*s6pdh*) for 1 min, and extension at 72°C for 2 min.

Following electrophoresis we excised PCR products from 1% agarose gels and purified them using a QIAquick Gel Extraction Kit (Qiagen). We sent the fragments for direct sequencing using the PCR primers either to Davis Sequencing (Davis, CA) or the DNA Sequence Laboratory of the University of Wisconsin Biotechnology Center (Madison, WI). PCR products with unreadable sequences or a significant number of ambiguous base calls were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen) following the manufacturer's instructions. Sequencing of all clones using T3 and T7 primers was done by the DNA Sequence Laboratory of the UW Biotechnology Center. Following cloning, sequences with possible PCR artifacts were identified and removed from further analysis by comparing the cloned sequences to each other and to the original chromatogram obtained through direct sequencing. When two or more clones yielded identical sequences, only one was used in the analyses.

Each sequence chromatogram was scrutinized for base determination errors using Chromas 2.22 (Technelysium Pty Ltd), and the sequences were aligned by eye in GeneDoc 2.6.002 (Nicholas & Nicholas 1997). The close relationship among *Prunus* species made manual alignment of the sequences straightforward. For data analysis, parsimony informative indels were coded as additional binary characters. Seven indels were included for *LEAFY* and eight for *s6pdh*. *LEAFY* sequences were trimmed to just Intron 2; *s6pdh* sequences were truncated to begin 18 bp downstream from the end of primer k, located in Exon 2, and end 14 bp upstream from the start of primer p in Exon 6. Sequences were submitted to GenBank (accession numbers listed in Table 1).

Phylogenetic Analyses.—Both data sets were analyzed using maximum parsimony (MP) with PAUP* 4.0b10 (Swofford 2002) and Bayesian inference (BI) with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsebeck 2003). Two heuristic MP searches with TBR branch swapping and the Multrees option were performed: the first with MaxTrees set to one million; in the second, the "nchuck" and "chuckscore" options were employed so that 10,000 trees were saved and swapped for each of 30 random addition replicates. The results of each search were summarized as strict consensus cladograms. Relative support for each branch was assessed by heuristic searches of 1000 bootstrap replications with 10 random addition replicates each and TBR branch swapping. For each random addition replicate 100 trees were saved. For BI analyses each data set was partitioned into nucleotide characters and indel characters. ModelTest 3.7 (Posada & Crandall 1998) was used to select the substitution model that best fit the nucleotide data using the Akaike information criterion: TVMef for *LEAFY* and HKY+I+G for *s6pdh*. Each of two BI runs for each gene consisted of three heated and one cold chain with samples drawn every 100 generations. We ran the *LEAFY* data for 1,000,000 generations discarding the first 500,000 generations, whereas for *s6pdh* we ran 1,500,000 generations discarding the first 750,000 generations. The runs for each data set were combined and a majority rule consensus tree constructed with a 67% threshold.

Determination of chloroplast haplotypes.—In order to compare our phylogenetic results from nuclear *LEAFY* and *s6pdh* with those of Shaw and Small (2004, 2005), we sequenced the *trnL-trnF* intergenic spacer from the chloroplast genome for our accessions. We amplified the spacer using the e-f primer pair of Taberlet et al. (1991) and used primer e, and sometimes primer f, for sequencing (GenBank accessions EU606152–EU606192). We aligned our sequences with those from the 18 *Prunocerasus* exemplars used in Shaw and Small

(2004). The maximum parsimony tree from the combined dataset was used to place each of our native plum accessions into one of the primary chloroplast haplotypes (A, B, C, S, or T) of Shaw and Small (2005).

RESULTS

For clarity in the results and discussion sections we refer to specific sequences by their names on the phylograms, omitting the genus name and differentiating among clones from the same collection by decimels following the last three digits of the collection number.

LEAFY Alignment and Data Analysis.—PCR with primers LFY5 and LFY4 yielded fragments approximately 750 or 1000 nucleotides in length, the difference being a 250-bp insertion. Only 1000-bp fragments were PCR amplified for *Prunus hortulana* and *P. murrayana*. All three individuals of *P. rivularis* and two of the four collections of *P. munsoniana* (513, 676) had both 750 and 1000-bp fragments (Fig 1). The other collections of *P. munsoniana* and the remainder of the species yielded only 750-bp fragments. Some of the cloned *P. rivularis* sequences appeared to be recombinations of the 750 and 1000-bp sequences, similar to those of *P. angustifolia* and *P. hortulana/P. murrayana* respectively (Fig 2). Putative recombinant sequences were omitted from the phylogenetic analyses. Trimmed to *LEAFY* Intron 2, the ingroup sequences varied in length from 658 bp in *P. texana* to 925 bp in one clone of *P. rivularis* (528.22). The final alignment used in the *LEAFY* data analysis included 930 nucleotide characters, of which 856 were invariant, 35 varied but were parsimony-uninformative, and 39 (4.2%) were parsimony informative for the ingroup taxa.

The strict consensus MP cladograms from the two parsimony analyses are identical (not shown), each of length 194 with consistency index (CI) of 0.634 and retention index (RI) of 0.796. Their topologies are similar to the Bayesian analysis phylogram (Fig. 3), but several nodes (indicated by the solid circles) are collapsed and there are minor rearrangements within Clades 3 and 4 (topology indicated by dotted lines). Species of *Prunus* sect. *Prunocerasus* form a monophyletic group split into three lineages: *P. subcordata* plus Clades 1 and 2. Clade 3 is composed of *P. angustifolia* and *angustifolia*-like sequences from *P. munsoniana* and *P. rivularis*. The latter two species (names in bold on Fig. 3) also have cloned sequences from the same individuals on Clade 2. Relationships among *P. americana*, *P. mexicana*, and *P. nigra* are unresolved; they diverge from a basal polytomy on Clade 2. Sequences of Clade 4, including those of *P. hortulana*, *P. murrayana* (except 525S), *P. munsoniana*, and *P. rivularis*, have a 250-bp insertion not found among the other sequences.

s6pdh Alignment and Data Analysis.—The final alignment for the *s6pdh* analysis includes 1272 nucleotide characters, of which 1197 were invariant, 46 varied but were parsimony-uninformative, and 29 (2.3%) were parsimony informative for the *Prunus* sect. *Prunocerasus* species. The shortest sequences among the ingroup were those of *P. hortulana* (628), *P. maritima*, and *P. subcordata* at 1204 nucleotides, including 7 or 8 TC repeats in Intron 3, whereas one *P. americana* clone (505.2) had the longest sequence at 1256 nucleotides with 31 TC repeats.

Just as in the *LEAFY* analyses, the strict consensus MP cladograms from both parsimony analyses of *s6pdh* are identical (not shown) and have lengths of 153, consistency indices (CI) of 0.699, and retention indices (RI) of 0.854. The MP cladograms are nearly identical to the BI phylogram (Fig. 4). On the MP cladograms, Clades 5 and 6 are sister groups such that *Prunus* sect. *Prunocerasus* forms a monophyletic group, albeit with bootstrap support less than 50%, the branch indicated by the black circle is collapsed, and *americana*.684.1 is sister to *americana*.495.3/*americana*.505.23 on Clade 5 (indicated by dotted lines). Clade 5 includes *P. subcordata* plus *subcordata*-like sequences from five individuals of *P. americana* and one of *P. munsoniana*. These accessions (names in bold on Fig. 4) also have sequences on Clade 6, which includes all the other native plum taxa. There is poor resolution among these taxa. *Prunus angustifolia*, *P. texana*, and *angustifolia*-like sequences from *P. munsoniana* and *P. rivularis* compose Clade 7. Clade 8 consists solely of *P. americana* and *P. mexicana*, plus a similar sequence from one individual of *P. umbellata* (520.4), which also has a sequence among the taxa of the Clade 6 basal polytomy (*umbellata*.520.3).



Fig. 1. Gel following electrophoresis of PCR products using LFY 5 and LFY 4 primers.

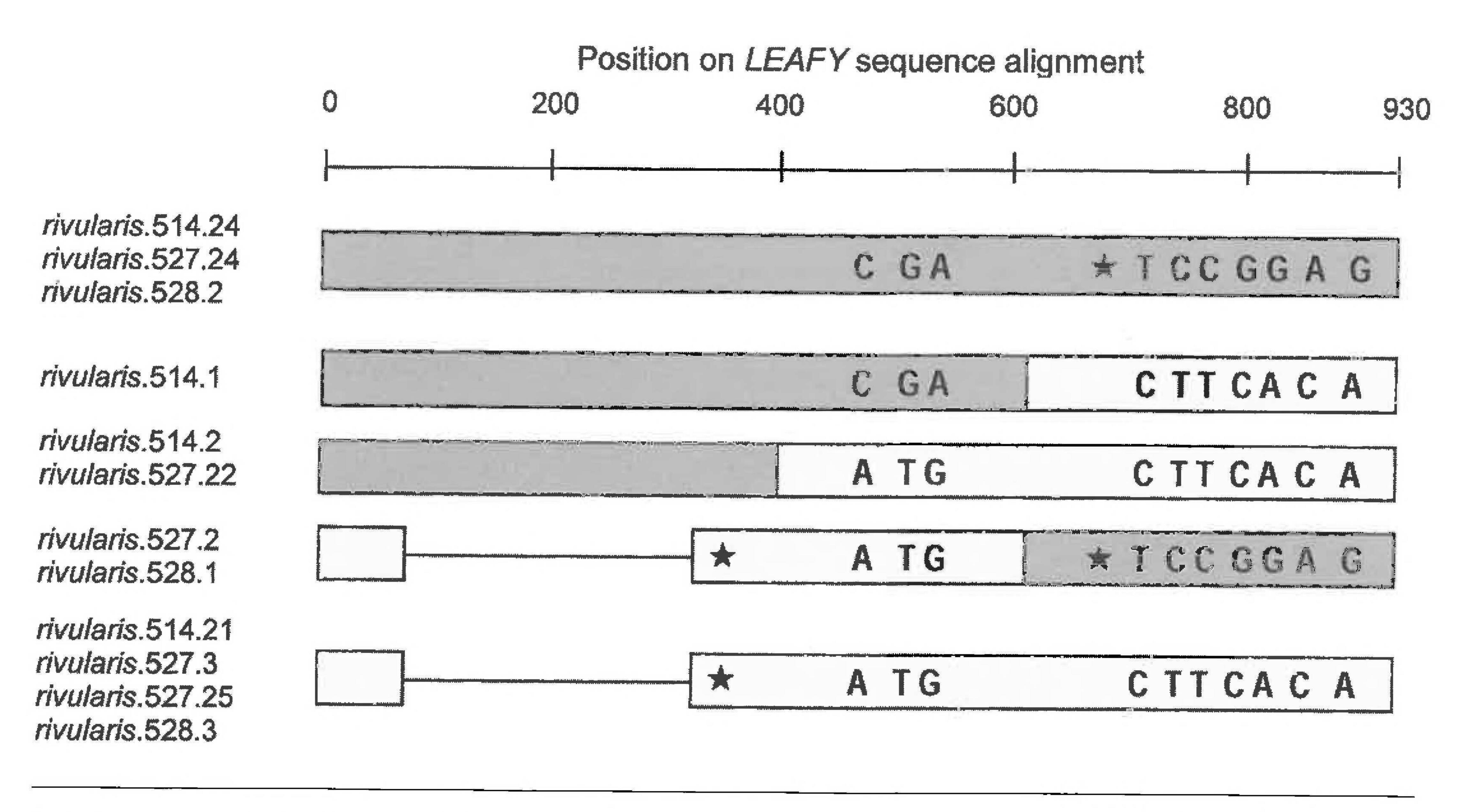


Fig. 2. Graphic representation of cloned *LEAFY* sequences from *Prunus rivularis* accessions. Each bar represents DNA sequence. Dark gray segments correspond to close relationship with *P. hortulana* and *P. murrayana* sequences (Clade 4 on Fig. 3) and light gray segments correspond to close relationship with *P. angustifolia* sequences (Clade 3 on Fig. 3). Characters used to map sequence segments: straight line indicates absence of 250-bp insertion (alignment gap), stars indicate the presence of 6-bp and 9-bp deletions, and letters indicate key nucleotide differences between the *hortulana*-type and *angustifolia*-type sequences. The boundaries between sequence types are drawn to within about 75 bp.

DISCUSSION

The nuclear genes *LEAFY* and *s6pdh* provide weak resolution of relationships among species of *Prunus* sect. *Prunocerasus*. The majority of taxa diverge from polytomies on the MP consensus and BI trees. The topology of these trees is different from the cpDNA cladogram of Shaw and Small (2005) where *P. subcordata* and then *P. texana* are sister groups to the remaining species, which segregate into three primary clades: American

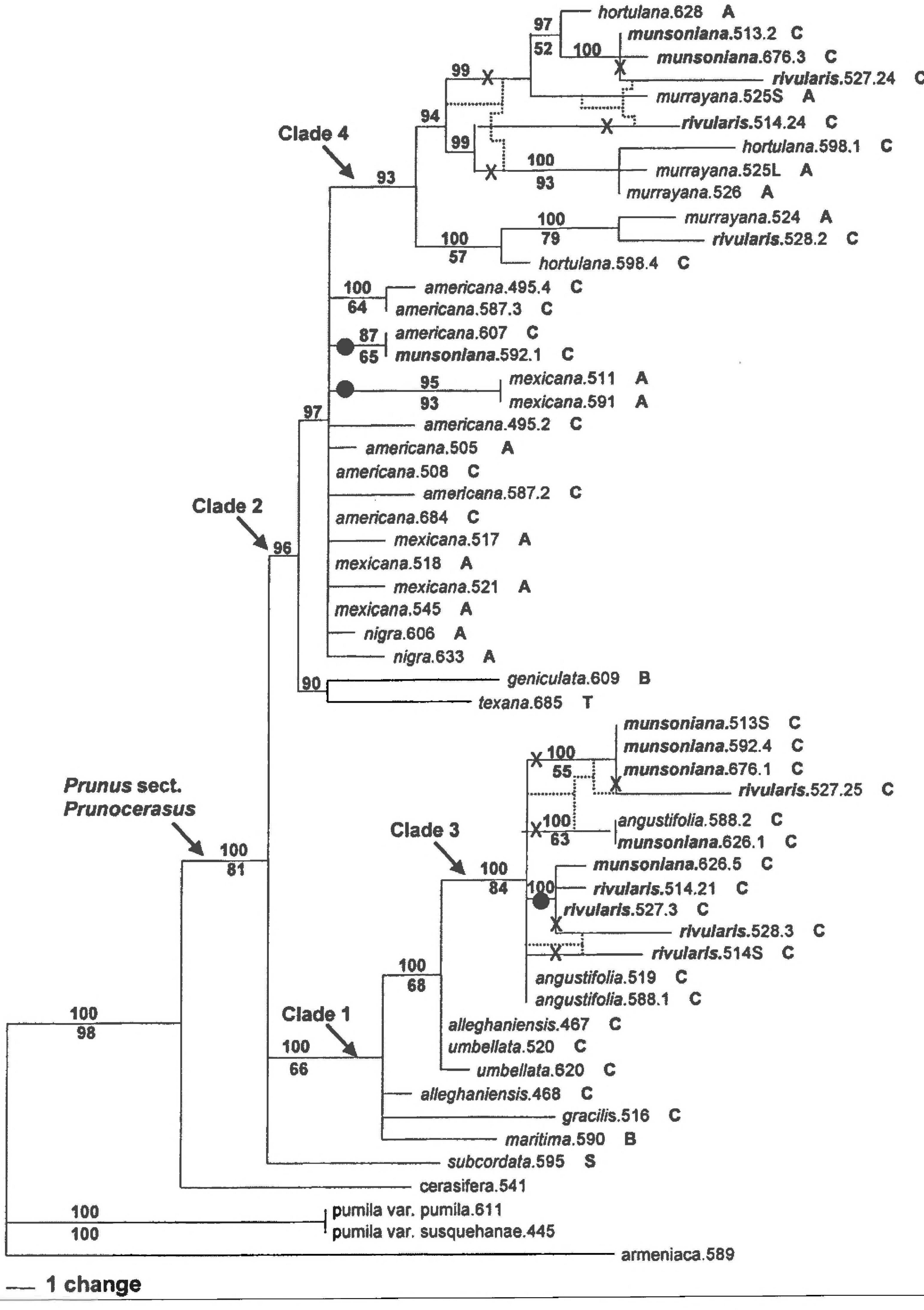


Fig. 3. LEAFY 67% majority rule consensus phylogram from Bayesian analysis. Ingroup taxa are italicized; outgroup taxa are in roman type. Support values above the branches are the BI posterior probabilities (%) for each clade; below branches are MP bootstrap values over 50%. MP strict consensus cladograms differ where black circles indicate branches that collapse, and branches cut at X's are reconnected as shown by dotted lines. Taxa are labeled by epithet plus last 3 digits of collection number (and clone number). Letters following the taxon names correspond to cpDNA haplotypes of Shaw and Small (2005) as determined from trnL-trnF spacer sequences.

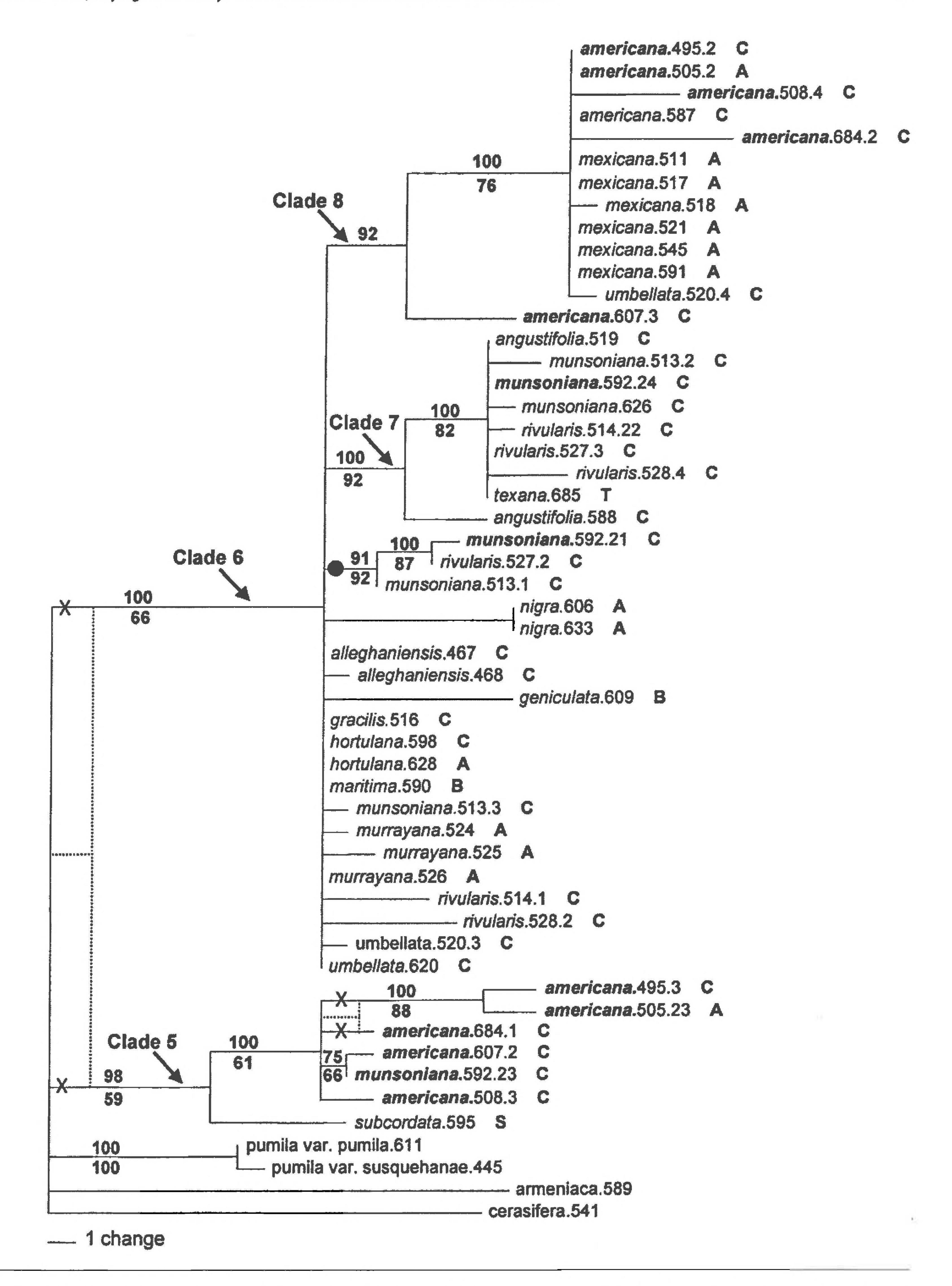


Fig. 4. s6pdh 67% majority rule consensus phylogram from Bayesian analysis. Ingroup taxa are italicized; outgroup taxa are in roman type. Support values above the branches are the BI posterior probabilities (%) for each clade; below branches are MP bootstrap values over 50%. MP strict consensus cladograms differ where black circle indicates branch that collapses, and branches cut at X's are reconnected as shown by dotted lines. Taxa are labeled by epithet plus last 3 digits of collection number (and clone number). Letters following the taxon names correspond to cpDNA haplotypes of Shaw and Small (2005) as determined from trnL-trnF spacer sequences.

(A), Beach (B), and Chickasaw (C). Clade 1 on the *LEAFY* tree and *s6pdh* Clades 5 and 7 consist largely of accessions with cpDNA haplotype C, otherwise taxa on the nuclear gene trees do not resolve into the A, B, and C clades of Shaw and Small (2005). Incongruence between chloroplast and nuclear phylogenies is common and typically attributed to such factors as hybridization, lineage sorting, or long-branch attraction (Albaladejo et al. 2005; Doyle 1997; Maddison 1997; Sang & Zhong 2000). The geographic distribution of the chloroplast ABC clades suggests introgression with chloroplast capture given the ease with which North American plums are known to hybridize (Hedrick 1911; Shaw & Small 2004).

Although *Prunus texana*, known as peachbush or Texas wild peach, has historically been placed in sect. *Amygdalus* due to its hairy-skinned fruit, Shaw and Small (2004) found that it has a plum chloroplast genome. Evidence from the nuclear genes *LEAFY* and *s6pdh* confirms its placement in sect. *Prunocerasus*. On the cpDNA cladogram *P. texana* was sister to the other plums of eastern North America, a position we could not confirm. It is sister to *P. geniculata*, the rare endemic of the Lake Wales Ridge in central Florida, on our *LEAFY* phylogram. However, this relationship is based on one character change and has weak bootstrap support. On the *s6pdh* phylogram *P. texana* is part of Clade 7 with *P. angustifolia* and the *angustifolia*-like sequences of *P. munsoniana* and *P. rivularis*.

Prunus alleghaniensis, P. gracilis, P. maritima, and P. umbellata form a paraphyletic group on LEAFY Clade 1, basal to Clade 3. These four species clustered on the UPGMA dendrogram based on similarity of microsatellite markers in the study of Rohrer et al. (2004). These species closely resemble each other morphologically and were allied by early students of American plums such as Wight (1915). On the s6pdh cladogram, they are part of the large Clade 6 basal polytomy.

Prunus americana and P. mexicana can be difficult to separate morphologically, especially when working with herbarium specimens. Similarly their LEAFY and s6pdh sequences were similar and without resolution into monophyletic P. americana and P. mexicana groups. On the LEAFY phylogram the P. americana and P. mexicana sequences diverge from a basal polytomy along with P. nigra, munsoniana.592.1, and Clade 4. LEAFY sequences americana.508 (WI), americana.684 (PA), mexicana.518 (TX), and mexicana.545 (cult.) differ trivially by the length of a poly-A run and some nucleotide characters heterozygous for one or two sequences but with only one of the two bases on the other sequences. Otherwise they are identical.

Although our data do not separate *Prunus americana* from *P. mexicana* sequences on either *LEAFY* Clade 2 or *s6pdh* Clade 8, Boonprakob et al. (2001) calculated a UPGMA dendrogram, based on similarities of RAPD markers, on which all 13 of their accessions of *P. americana* clustered together and distinct from a cluster of all 11 accessions of *P. mexicana*. Clearly more collections with a wide geographic distribution and more genes will need to be examined and correlated with the morphological characteristics of those accessions before the taxonomic structure of the *P. americana-mexicana* complex is understood.

In an earlier microsatellite study, Rohrer et al. (2004) included two collections from east Texas near Marshall (designated P. sp. (americana x mexicana?) 10517 and 10518 in that publication) that are morphologically intermediate between typical Prunus americana and P. mexicana. The lack of differentiation between P. americana and P. mexicana LEAFY and s6pdh sequences leaves their determination ambiguous. However, because neither 517 nor 518 have a subcordata-like s6pdh sequence and because most modern floras exclude P. americana from Texas, we have provisionally determined these accessions to be P. mexicana.

Two distinct sequences were isolated from five of the six accessions of *Prunus americana* by cloning their s6pdh PCR products. One sequence from each pair is on Clade 8 along with *P. mexicana* and the single sequence of *P. americana* 587; the other is on Clade 5 with *P. subcordata*. Given that these *P. americana* sequences form a monophyletic group sister to *P. subcordata* and that *P. subcordata* grows only in California and Oregon, far west of *P. americana* range in eastern North America, it seems unlikely that the five *P. americana* collections with two s6pdh sequences are recent hybrids independently involving *P. subcordata*. If s6pdh is a single copy gene in all diploid *Prunus* species as concluded by Bortiri et al. (2002) and if *P. americana* is diploid as it is reported to be (2n = 16, L"ove & L"ove 1982), then a high degree of heterozygosity is being maintained at a single locus. However, we cannot rule out the possibility that there are two heterologous

ancestral sopth loci across Prunus with only one or the other copy observed in most North American plums, or alternatively gene duplication in P. americana. Bortiri et al. (2002) found divergent sopth sequences in Prunus caroliniana and P. emarginata. They hypothesized that divergent paralogs in P. caroliniana, a tetraploid species, may have arisen through gene duplication associated with polyploidization. In P. caroliniana the inferred polypeptides of the divergent clones differed at 16 of 251 amino acids (Bortiri et al. 2002), whereas for each of our five accessions of Prunus americana with divergent sopth sequences, the inferred polypeptides of the two clones differ by only 1–4 out of 223 amino acids. To better explain the existence of divergent sequences in P. americana, it would be useful to have chromosome counts from each of those accessions to verify that they are indeed diploid. Also because Southern blotting with a Malus domestica cDNA sopth probe by Bortiri et al. (2002) detected more than one region of sequence similarity in several genera of Rosaceae, the possibility of paralogous genes might be further investigated.

Alleles closely related to those of *Prunus americana*, and possibly *P. mexicana*, were found in other plum species suggesting introgression. *Prunus munsoniana* 592 has both s6pdh and *LEAFY* sequences monophyletic with sequences from *P. americana* 607. On the s6pdh tree munsoniana 592.23 is nested with the five subcordatalike *P. americana* clones and has the same amino acid sequence as americana.495.3 and americana.505.23. On the *LEAFY* tree munsoniana.592.1 is sister to americana.607. Also *P. umbellata* 520 has an americana/mexicana-type s6pdh sequence (umbellata.520.4) in addition to a sequence similar to that of the *P. umbellata* 620 collection.

Sequences from both LEAFY and sopplh suggest that Prunus rivularis may be an allopolyploid with P. angustifolia as one parent. The LEAFY sequences further suggest that the other parent is most likely P. hortulana or P. murrayana and that recombination between parental sequences has taken place. Three or four distinct sequences were cloned from each of the P. rivularis accessions. LEAFY is believed to be a single-copy nuclear gene (Frohlich & Parker 2000; Oh & Potter 2003) and would be expected to have one or two alleles in a diploid species. At least one sequence from each P. rivularis accession is monophyletic with sequences of P. angustifolia (Clade 3 on the LEAFY phylogram and Clade 7 on the s6pdh phylogram). The other sequence is on Clade 4 of LEAFY along with sequences of P. hortulana and P. murrayana. In addition, some LEAFY recombinant sequences were cloned where part of the sequence matches P. angustifolia and the rest matches P. hortulana/murrayana (graphically illustrated on Fig. 2). Although it is possible for recombinant sequences to arise as PCR artifacts (Cronn et al. 2002; Posada et al. 2002), in our study they were isolated only from P. rivularis and they were found among the LEAFY sequences of all three P. rivularis accessions. This would seem to support the hypothesis that the recombinant sequences arose through meiotic recombination following hybridization between P. angustifolia and either P. hortulana or P. murrayana. Given the few phylogenetically-informative characters in the s6pdh data, if there were recombinant s6pdh sequences, we were unable to identify them.

Plants in our study determined as *Prunus munsoniana* appear to be a mixture of hybrids having *P. angustifolia* as one of the parents. All four *P. munsoniana* collections have a *LEAFY* sequence on Clade 3 with *P. angustifolia* and *P. rivularis*, and three collections have s6pdh sequences on Clade 7, again with *P. angustifolia* and *P. rivularis* (we are lacking a s6pdh sequence for *P. munsoniana* 676). Two *P. munsoniana* collections (513 and 676) have a second *LEAFY* sequence with the 250-bp insertion found also among sequences of *P. hortulana*, *P. murrayana*, and *P. rivularis* on Clade 4. On the BI cladogram, *munsoniana*.513.2 and *munsoniana*.676.3 form a trichotomy with *rivularis*.527.24, but on the MP cladogram they are sister to *hortulana*.628. Thus, these two *P. munsoniana* collections have *LEAFY* sequences closely related to those of *P. rivularis*, which lends support to the suggestion by Diggs et al. (1999) that *P. munsoniana* is simply a larger version of *P. rivularis*. However, the other two specimens of *P. munsoniana* (592 and 626) lack *hortulana/murrayana*-like *LEAFY* sequences, yet are very similar morphologically to 513 and 676. For *P. munsoniana* 626 we were only able to isolate *angustifolia*-like *LEAFY* sequences. Likewise we isolated only a single s6pdh sequence, which lies on Clade 7 with *P. angustifolia* and *P. rivularis*. *P. munsoniana* 513 and 592 each yielded three distinct s6pdh sequences, suggesting that at least some *P. munsoniana* specimens may be allopolyploids. *P. munsoniana* 592

has a sequence on s6pdh Clade 7 (592.24) with *P. angustifolia*, and on Clade 5 (592.23) sister to a clone of *P. americana* (607.2), just as on the *LEAFY* phylogram. The third s6pdh sequence (592.21) is sister to rivularis.527.2 and these two are sister to munsoniana.513.1.

To our knowledge we are the first to suggest a close phylogenetic relationship between Prunus murrayana and P. hortulana (Clade 4 on the LEAFY phylogram). P. murrayana.525L and murrayana.526 form a clade with hortulana.598.1 having 93% MP bootstrap support and 100% BI posterior probability. The s6pdh sequences do not confirm or refute the close relationship between P. murrayana and P. hortulana, because their sequences along with those of P. alleghaniensis, P. geniculata, P. gracilis, and several other species all are unresolved on the Clade 6 basal polytomy. Until a decade ago, P. murrayana was a poorly known species endemic to Texas west of the Pecos River and represented in herbaria by only a handful of specimens. When originally described, Palmer (1929) wrote that it might be most closely related to P. rivularis. Enquist (1997) formalized this relationship by designating P. murrayana as a synonym when he described P. rivularis var. pubescens Enquist. He discovered numerous plums on the western side of the Edwards Plateau with pubescent first-year branchlets, pubescent pedicels, and pubescence encircling the petioles, and he concluded that these plants were the same taxon as P. murrayana known from further west. Many of Enquist's collections are morphologically well matched with authentic P. murrayana specimens. He explained how most of the apparent differences between P. murrayana and P. rivularis did not hold in light of the new collections and that the only distinguishing character was pubescence; the branchlets, pedicels, and petioles of P. rivularis are glabrous or nearly so (Enquist 1997). Clade 4 of the LEAFY phylogram includes not only P. hortulana and P. murrayana but also sequences from all three accessions of P. rivularis and a couple from P. munsoniana. Our P. murrayana collections are from Coke and Tom Greene Counties east of the Pecos River at localities where Enquist (1997) collected P. rivularis var. pubescens [= P. murrayana]. These collections have only Clade 4 LEAFY sequences, whereas our three collections of P. rivularis have both Clade 4 and Clade 3 sequences. Similarly, P. murrayana accessions have sopul sequences only from the Clade 6 polytomy, whereas all three P. rivularis accessions have a cloned sequence on Clade 7 with P. angustifolia and from the basal polytomy of Clade 6. We believe that P. murrayana is a diploid species distinct from P. rivularis, which we believe is an allopolyploid derived from P. angustifolia and either P. murrayana or P. hortulana.

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